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Preparation and characterisation of chitosans with oligosaccharide branches

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Abstract

The trimer 2-acetamido-2-deoxy-D-glucopyranosyl-β-(1→4)-2-acetamido-2-deoxy-D-glucopyranosyl-β-(1→4)-2,5-anhydro-D-mannofuranose (**A**–**A**–**M**) was reductively *N*-alkylated onto a fully de-*N*-acetylated chitosan ($F_A < 0.001$, $DP_n = 25$) to obtain branched chitosans with degree of substitution (DS) of 0.070, 0.23 and 0.40, as determined by ^{1}H NMR spectroscopy. The apparent pK_a values of the primary and secondary amines of the chitosans substituted with the trimer **A**–**A**–**M** were determined by monitoring the chemical shift of the H-2 of GlcN, and were determined as 6.5–6.9 for the primary (unsubstituted) amines and as 5.0–5.2 for the secondary (substituted) amines. The intrinsic pK_a values (pK_{int}) were found to be 7.3–7.4 for the substituted and 8.7 for the unsubstituted amines. The chitosan branched with **A**–**A**–**M** (DS 0.40) was found to be soluble in aqueous solution over the entire pH range. SEC-MALLS (size-exclusion chromatography with a multi-angle laser light scattering detector) further showed that addition of branches did not affect the molar hydrodynamic volume of the chitosan.

Keywords: Chitosan; Reductive alkylation; Oligosaccharide branches; ^{1}H NMR; SEC-MALLS

1. Introduction

Chitosan is a linear polysaccharide composed of β-(1→4)-linked 2-amino-2-deoxy-D-glucopyranose (GlcN, **D**-unit) and 2-acetamido-2-deoxy-D-glucopyranose (GlcNAc, **A**-unit). Chitosan is produced industrially by alkaline de-*N*-acetylation of chitin, a structural polysaccharide in the exoskeleton of arthropods.[1] In commercial chitosans, the fraction of **A**-units (F_A) may usually range from 0 to 0.3. Structure–function relationships in chitosans with different chemical composition (F_A) have revealed considerable differences between chitosans, e.g., solubility as a function of pH,[2] binding to lysozyme,[3,4] degradation by lysozyme,[5–7] drug delivery[8] and recently in gene delivery.[9] Studies of the functional properties of the branched chitosans are currently in progress.

Generally, chitosans are only soluble in water when the free amino groups are protonated. The pK_a value of the primary amines of chitosan has been determined to be around 6.5[10–12] and most high molecular weight chitosans become insoluble at physiological pH values (7.2–7.4). Strategies to increase the neutral-solubility of chitosans include classical chemical derivatisation (e.g., carboxymethylation,[13] sulfatation[14]) or by preparing chitosans with F_A near 0.50.[15] Alternatively, various types of branches may be introduced, which generally enhances solubility.

Several studies on the covalent attachment of aldehydes to chitosan by the use of reductive *N*-alkylation have been reported. Yalpani and Hall[16] performed an extensive study on branching chitosan with monosaccharides, oligosaccharides and dextrans. Sugimoto et al.[17] prepared water-soluble chitin and chitosan derivatives by branching chitosan with variable length

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poly(ethylene glycol)-aldehydes and subsequent *N*-acetylation. Kurita, Amemiya, Mori and Nishiyama[18] prepared comb-shaped chitosan derivatives having oligo(ethylene glycol) side chains showing higher affinity for organic solvents as well as water, in addition to improved adsorption capacity towards metal ions. Holme and Hall[19] observed temperature-induced gelation when they *N*-alkylated chitosan with a variable length alkyl-glycoside. Casu et al.[20] prepared chitosan branched with oligomers of the type **A**–[**A**]$_n$–**M**, similarly to those prepared in this work, but without a clearly defined chain length of the chitosan or side-chain, and the branched chitosans were only briefly characterised.

We recently reported on the preparation and characterisation of oligosaccharides with a reactive 2,5-anhydro-D-mannofuranose (**M**) unit at the new reducing end.[21] In this study we report on the grafting of the trisaccharide 2-acetamido-2-deoxy-D-glucopyranosyl-β-(1→4)-2-acetamido-2-deoxy-D-glucopyranosyl-β-(1→4)-2,5-anhydro-D-mannofuranose (**A**–**A**–**M**) onto a fully de-*N*-acetylated, low molecular weight chitosan by reductive *N*-alkylation. In addition, the same starting chitosan was also substituted with either acetaldehyde or D-glucose. The new chitosans were characterised by ^{1}H NMR spectroscopy and SEC-MALLS.

2. Experimental

Materials.—Two chitosans with fraction of *N*-acetylated units (F_A) of 0.59 and < 0.001 (as determined by ^{1}H NMR spectroscopy), were used in this study. Chitin was first prepared from shrimp shells as described by Hackmann.[22] The chitosan with $F_A = 0.59$ (intrinsic viscosity, $[\eta] = 826$ mL/g at pH 4.5 and ionic strength of 0.1 M) was prepared by homogeneous de-*N*-acetylation[23] of chitin, while the fully de-*N*-acetylated chitosan ($F_A < 0.001$, $[\eta] = 283$ mL/g) was prepared by further heterogeneous deacetylation of a commercial chitosan with $F_A = 0.01$. Sodium nitrite, ammonium acetate, sodium cyanoborohydride, acetaldehyde and D-glucose were obtained from Merck. D_2O (99.96% D atom) was purchased from Isotech Inc., Superdex 30 (prep. grade) came from Amersham Pharmacia Biotech, DCl (35%) and NaOD from Sigma. All dialyses in this study were performed with tubes from Medicell International Ltd. (MW cut-off when used for globular proteins: 12–14 kDa).

Preparation of fully N-*acetylated oligomers.*—The trimer 2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→4)-2,5-anhydro-D-mannofuranose (**A**–**A**–**M**) was prepared as earlier described[21] using a partially de-*N*-acetylated chitosan ($F_A = 0.59$, $[\eta] = 826$ mL/g) as starting material.

Preparation of low-molecular-weight fully de-*N*-a*cetylated chitosan.*—The low-molecular-weight fully de-*N*-acetylated chitosan was prepared by nitrous acid depolymerisation with a molar ratio of $NaNO_2$ to **D**-units of 0.10, followed by convential reduction by $NaBH_4$, dialysis and lyophilisation.[21,24] The chitosan was found to be fully reduced and the average number degree of polymerisation (DP_n) was determined to 25 by ^{1}H and ^{13}C NMR spectroscopy.

NMR spectroscopy.—Samples were dissolved in D_2O, and transferred to 5 mm NMR tubes. The NMR spectra were acquired on a Dpx 400 Bruker Avance spectrometer at 400.13 MHz and 25 °C for ^{1}H, and 100.63 MHz and 90 °C for ^{13}C. All chemical shifts were determined relative to the internal standard sodium 3-(trimethylsilyl)-propionate-d_4 (TSP) from Aldrich Chem. Co., 5 μL added from a 1% stock solution.[25] Typical conditions used for acquisition of the ^{1}H NMR spectra: size of spectral window was 8220 Hz; centre of the ^{1}H NMR spectra was 1880 Hz; acquisition time, 3 s; number of scans, 64; a 30° excitation pulse-angle was used; data size, 32 K. Conditions for acquisition of the DQF-COSY (double quantum filtrated homonuclear correlation spectroscopy) spectrum:[26] memory size, 1024 K (F2) × 128 K (F1); size of spectral window, 2400 Hz (F2) × 2400 Hz (F1); centre of spectrum, 1881 Hz. The COSY spectra were recorded in D_2O at 25 °C and pH* 5.0. The pH* (value obtained when measuring with a pH meter, corrected for isotope effect:[27] pD = pH* + 0.4) was measured by use of a Mettler Toledo pH electrode.

Branching reaction.—A solution of low-molecular-weight fully de-*N*-acetylated chitosan ($DP_n = 25$, 20 μmol **D**-units) and fully *N*-acetylated trimer (**A**–**A**–**M**) (2.0, 12, 20 and 40 μmol) in 0.1 M acetic acid with 0.1 M NaCl was allowed to react for 4 days (5 mL, pH 5.5, room temperature). $NaCNBH_3$ (50 mg) was added to the reaction mixture after 2 and 24 h, respectively. The pH during the reaction never exceeded 6.5. Remaining unreacted trimer (**A**–**A**–**M**) was removed by dialysis, and the branched chitosans were converted to the chloride salts, lyophilised and stored at −20 °C. Similarly, low molecular-weight fully de-*N*-acetylated chitosan ($DP_n = 25$, 20 μmol **D**-units) was also branched with acetaldehyde (4.0 μmol) and D-glucose (4.0 μmol) using the same procedure as for the fully *N*-acetylated trimer (**A**–**A**–**M**).

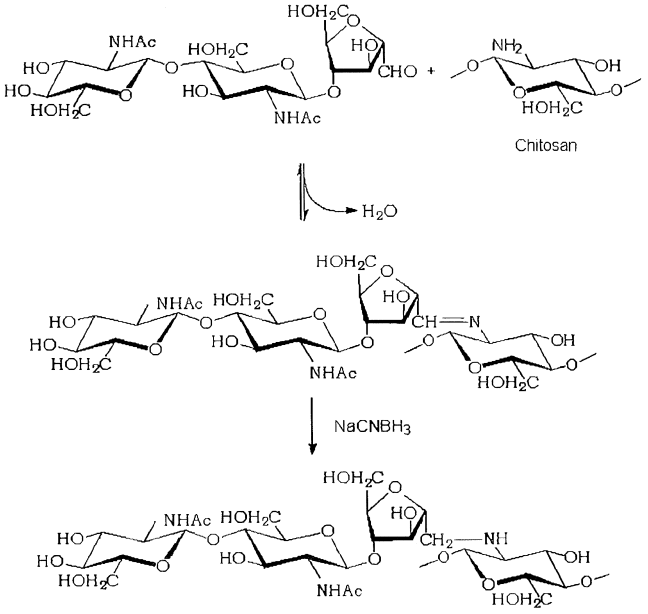
SEC-MALLS.—Size exclusion chromatography with refractive index (RI) and multi-angle laser light scattering detectors (SEC-MALLS) was used to study the molecular weight distributions of the branched chitosans. All samples were dissolved in deionised water (approx. 1 mg/mL) and clarified by filtration (0.80 μm) prior to injection (250–500 μL). Light scattering was monitored by a DAWN DSP multi-angle laser-light scattering detector. The RI-detector used was a DAWN Optilab 903. The refractive index increment (dn/dc) was

found to be of 0.142, for the acetate form (unpublished results). The system was eluted with 0.2 M ammonium acetate (pH 4.5) at a flow rate of 0.5 mL/min. Three HPLC-SEC columns connected in series were used: TSK 6000, 5000 and 4000 PWXL. The separations were performed at room temperature.

^{1}H NMR titration studies.—Chitosan substituted with the *N*-acetylated trimer (**A**–**A**–**M**) with DS = 0.070 and DS = 0.40, and chitosan substituted with acetaldehyde (DS = 0.20) and D-glucose (DS = 0.20) were dissolved (10 mg in 0.7 mL D_2O, 0.1 M NaCl) and transferred to 5 mm NMR tubes. Starting at low pD value (2.5), adjusted by 0.1 M DCl, a standard ^{1}H NMR spectrum was aquired. The pD was increased in a step-wise fashion using 0.1 M NaOD. After each adjustment, a new ^{1}H NMR spectrum was acquired. This process was continued until the sample either precipitated or to a pD above 10. The unsubstituted chitosan precipitated at pH 6.7, and the chemical shift of the primary amines in the other samples have been included in order to enable a more precise calculation of the pK_a value. An identical titration experiment was carried out for one of the samples (DS = 0.40) where D_2O was replaced with 90% H_2O/10% D_2O. The resulting titration curve was used to calculate a correction factor between pH and pD.

3. Results and discussion

Branching by reductive N-alkylation.—A fully de-*N*-acetylated chitosan ($F_A < 0.001$) was depolymerised to a number average degree of polymerisation (DP_n) of 25, as determined by NMR. The 2-amino groups were then reductively *N*-alkylated with the trimer 2-acetamido-2-deoxy-D-glucopyranosyl-β-(1→4)-2-acetamido-2-deoxy-D-glucopyranosyl-β-(1→4)-2,5-anhydro-D-mannofuranose (**A**–**A**–**M**) (Scheme 1). The use of a low-molecular-weight and fully de-*N*-acetylated chitosan to which the trimer **A**–**A**–**M** is covalently bound allows a precise determination of the degree of substitution (DS) by, e.g., ^{1}H NMR spectroscopy, even at low DS values. Furthermore, the enhanced resolution in the NMR spectrum of a relative low-molecular-weight chitosan allows more detailed assignment of the different protons. The pH value was kept between 5.5 and 6.5 during the reaction, both in order to avoid precipitation of the polymer, and to maintain reactivity of the 2-amino groups. The reduction compound $NaCNBH_3$ selectively reduces the imino bonds formed by Schiff base formation provided pH > 4.5.[28] Unreacted trimer (**A**–**A**–**M**) was removed by dialysis. Four different aldehyde-to-amino molar ratios ([**A**–**A**–**M**]/[GlcN]) were used: 0.10, 0.60, 1.0 and 2.0. The degrees of substitution were determined from the ^{1}H NMR spectra given in Fig. 1. The absence of Schiff bases (δ 8.1) and hydrated reducing **M**-ends (δ 5.01) in the spectra ensured that all trimers (**A**–**A**–**M**) in the sample were bound to the chitosan ($F_A < 0.001$) by covalent bonds to the 2-amino groups of the **D**-units (see Scheme 1).[21] The DS values were calculated from the methyl resonance of the *N*-acetyl group at δ 2.1 compared to H-2 of substituted and unsubstituted **D**-units at 2.5–3.3 ppm. In addition it was possible to assign the resonances of the H-2 protons of substituted and unsubstituted GlcN from the DQF-COSY spectrum (see Fig. 2), where the coupling of H-1 to H-2 is clearly seen. The assignment of the ^{1}H NMR spectrum is summarised in Table 2. Comparison to previous assignments of the ^{1}H NMR spectrum of chitosan[29,30] shows that the major changes in chemical shifts occur for protons near the branching point. In particular, C-1 of the **M**-unit has two stereochemically different protons at 3.63 and 4.06 ppm, when further linked to a **D**-unit. Similarly, ^{1}H NMR spectra of the chitosans ($F_A < 0.001$) branched with acetaldehyde (DS = 0.20) and D-glucose (DS = 0.20) revealed two different resonances (H-1/H-1*) at the branching point, and the chemical shift of these protons showed a strong dependency on the pH value, similar to the H-2 signal of the **D**-units (Fig. 3).



Scheme 1. Reductive amination of chitosan with the trimer **A-A-M** to obtain the branched chitosan.

Determination of pK_a values of the 2-amino groups.—Secondary aliphatic amines generally have higher pK_a values than the corresponding primary amines.[31] The pK_a values of the unsubstituted (primary) and the substituted (secondary) amines of two branched chitosans (substituted with the trimer **A**–**A**–**M**), DS 0.07 and 0.40 were determined by ^{1}H NMR (Fig. 1). H-2 of the substituted and unsubstituted **D**-units resonates at different chemical shifts, allowing their pK_a values to be determined from the change in chemical shift as a

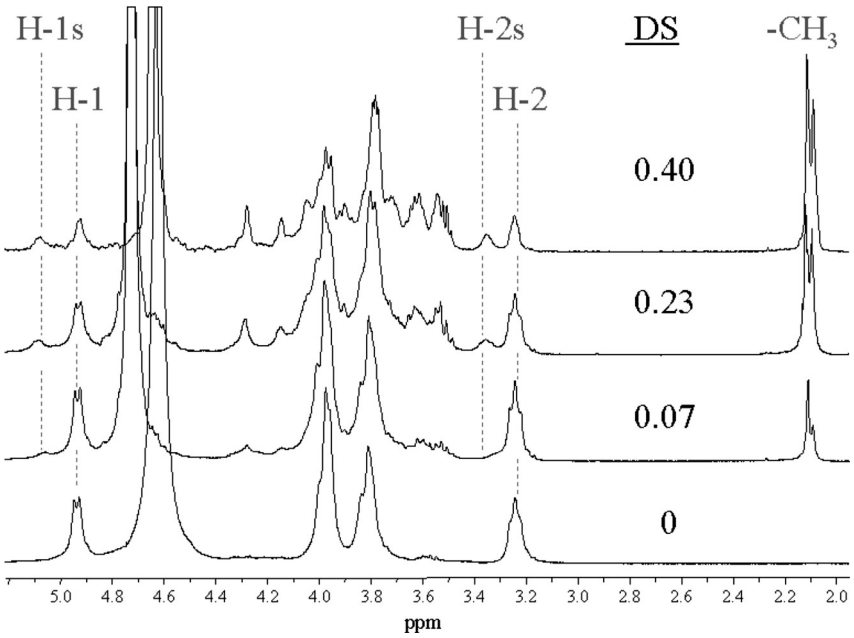


Fig. 1. ^{1}H NMR spectra of chitosan (DP_n 25, $F_A < 0.001$) *N*-alkylated with the *N*-acetylated trimer **A**–**A**–**M**. Conditions: 400.13 MHz, pH* 3–3.5 and 25–30 °C.

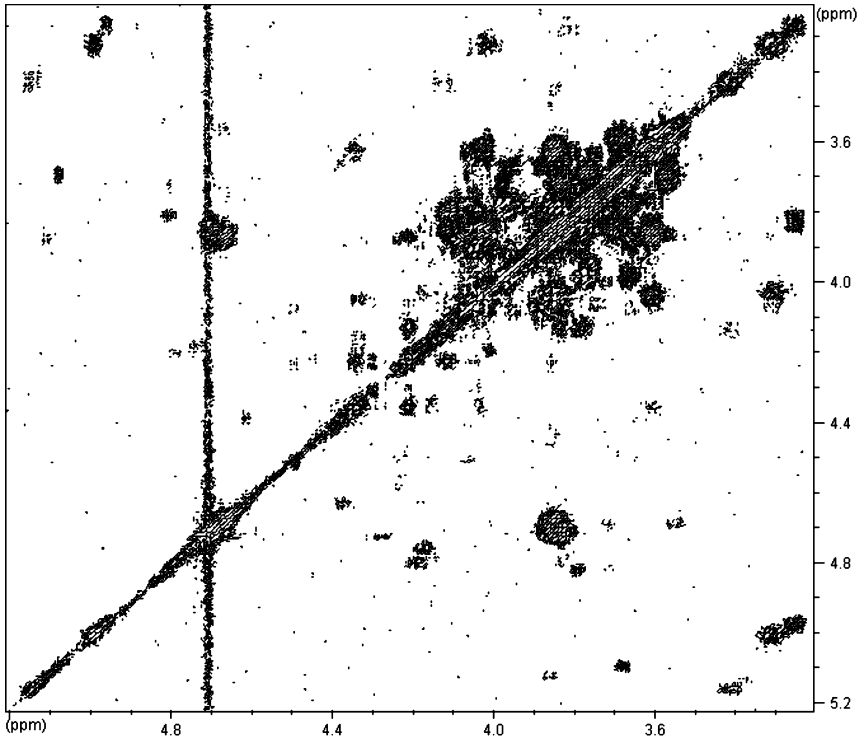


Fig. 2. DQF-COSY of chitosan (DP_n 25, $F_A < 0.001$) *N*-alkylated with the trimer **A**–**A**–**M** (DS 0.40). Conditions: 600 MHz, pH* < 3.5 and 30 °C.

function of pH. The corresponding titration curves are given in Fig. 3. To correct for the difference between pD and pH, a correction factor was calculated by performing an identical titration experiment for one of the samples (DS = 0.40) where D_2O was replaced with 90% H_2O/10% D_2O. According to the observed inflection points, the apparent pK_a values of the secondary amines were between 5.0 and 5.2, whereas those of the primary amines were determined to 6.6. The degree of substitution did not seem to influence the acidity of the

primary amines, as the pK_a values of the unsubstituted chitosan and the chitosans substituted with trimer (DS of 0.070 and 0.40) were similar. Moreover, the pK_a values of the secondary amines of the chitosans substituted with trimer with DS of 0.070 and 0.40 were determined to 5.0 and 5.2, respectively, suggesting that the DS was not important to the acidity of the secondary amines.

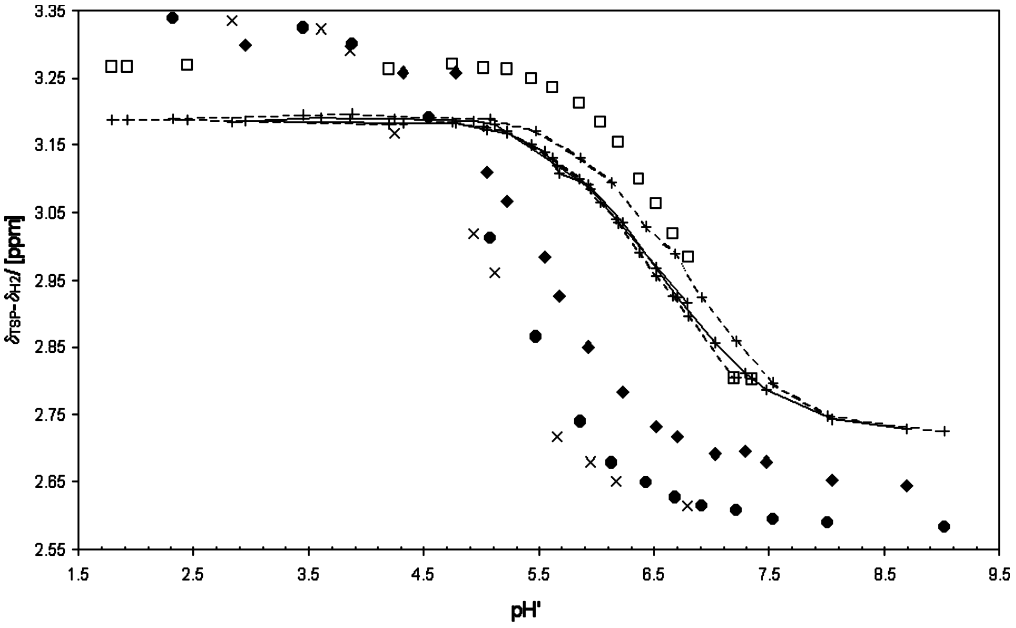


Fig. 3. Titration curves for the H-2 resonance of the substituted and unsubstituted 2-amino groups of the chitosans obtained by ^{1}H NMR in 0.1 M NaCl and 99.6% D_2O at 25 °C (10 mg/mL). Correction for isotope effect: pH′ = pH* − 0.2 as calculated from an identical experiment performed in 90% H_2O for one of the samples (DS 0.40). Chemical shift of H-2 resonance of the unsubstituted 2-amino groups of chitosan substituted with: (--+--) acetaldehyde (DS = 0.20), (——+——) glucose (DS = 0.20), (·····+·····) **A**–**A**–**M** (DS = 0.07), (– – +– –) **A**–**A**–**M** (DS = 0.40). Chemical shift of H-2 resonance of the substituted 2-amino groups of chitosan substituted with: (□) acetaldehyde (DS = 0.20), (◆) glucose (DS = 0.20), (×) **A**–**A**–**M** (DS = 0.07), (●) **A**–**A**–**M** (DS = 0.40).

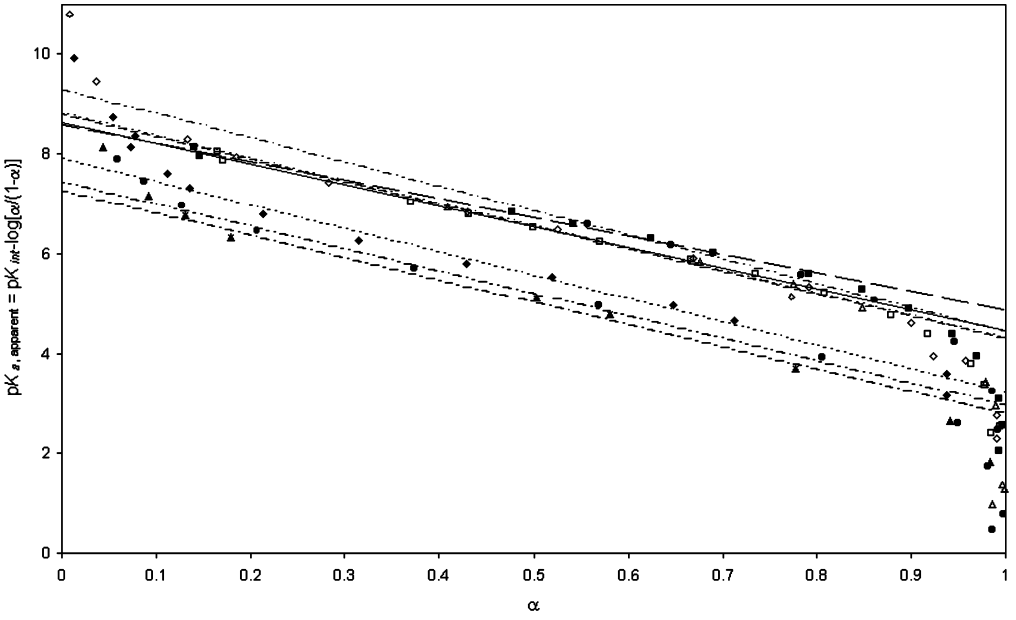


Fig. 4. Plot of the apparent dissociation constant (pK_a) for the unsubstituted and the substituted 2-amino groups of the chitosans as a function of the ionization degree (α) according to Katchalsky,[32] based on the data in Fig. 3. Unsubstituted 2-amino groups of chitosan substituted with: (□) acetaldehyde (DS = 0.20), (◇) glucose (DS = 0.20), (△) **A**–**A**–**M** (DS = 0.07), (○) **A**–**A**–**M** (DS = 0.40). Substituted 2-amino groups of chitosan substituted with: (■) acetaldehyde (DS = 0.20), (◆) glucose (DS = 0.20), (▲) **A**–**A**–**M** (DS = 0.07), (●) **A**–**A**–**M** (DS = 0.40).

Table 1
Molar ratio of the reacting groups, degree of substitution (DS), dissociation constant (pK_a) and the intrinsic dissociation constant (pK_{int}) for the substituted and unsubstituted 2-amino groups of the **D**-units and solubility in water (0.1 M NaCl) below/above pH 6.7 of the branched chitosans (n.d.: not determined)

Substituent *N*-alkylated on the 2-amino group	[Aldehyde]/[GlcN]	DS[a] (mol fraction)	pK_a[b] ($\alpha = 0.5$)		pK_{int}[b] ($\alpha = 0$)		Solubility pH < 6.7/pH > 6.7
			-NHR	$-NH_2$	-NHR	$-NH_2$	
Trimer **A-A-M**	0.10	0.070	5.0	6.6	7.3	8.8	+/−
	0.60	0.23	n.d.	n.d.	n.d.	n.d.	+/n.d.
	1.0	0.40	5.2	6.9	7.4	9.3	+/+
	2.0	0.40	n.d.	n.d.	n.d.	n.d.	+/n.d.
D-Glucose	0.20	0.20	5.6	6.6	7.9	8.8	+/−
Acetaldehyde	0.20	0.20	6.6	6.5	8.4	8.6	+/−
Unsubstituted[c] GlcN($-NH_2$)	–	–	–	6.6	–	8.7	–

[a] Determined from ^{1}H NMR after dialysis.
[b] Determined from the Katchalsky plots in Fig. 4.
[c] Average over all unsubstituted amino groups in the four titration experiments, see Figs. 3 and 4.

Table 2
Chemical shifts determined for chitosan ($F_A < 0.001$, $DP_n = 25$) branched with the trimer (**A**–**A**–**M**) (DS = 0.40) from the DQF-COSY-gs spectrum in Fig. 2 (pH* 3.0, 30 °C)

Proton observed	**D**-unit unsubstituted	**D**-unit substituted	**M**-unit (branching-point)
H-1/H-1′*	4.98	5.13	3.63/4.06
H-2	3.32	3.42	4.35
H-3	3.82	4.04	4.22
H-4	n.d.	n.d.	4.13
H-5	n.d.	n.d.	n.d.
H-6/H-6′	n.d.	n.d.	n.d.

Determination of intrinsic pK_a values (pK_{int}) of the 2-amino groups.—In Fig. 4, the apparent pK_a values are presented as a function of the degree of ionisation (α) as calculated from the ^{1}H NMR titration data given in Fig. 3, according to the Katchalsky equation.[32]

$$-\log[\alpha/1-\alpha] + \mathrm{pH} = \mathrm{p}K_{\mathrm{int}} + \phi(\alpha)\cdot\alpha = (\mathrm{p}K_a)_{\mathrm{apparent}}$$

where α is the ionisation degree, pK_{int} is the intrinsic pK_a corresponding to an uncharged polymer, $\phi(\alpha)$ is the electrostatic potential involved in moving an ion from a reference state at infinite distance to the surface of the polymer.[33] At $0.1 < \alpha < 0.8$, the apparent pK_a values decreased proportionally with increasing charge density, and a straight line could be fitted by regression (see Fig. 4). Extrapolation of the data to zero chargedensity ($\alpha = 0$) gave the pK_{int} values as presented in Table 1. The pK_{int} value for the primary (unsubstituted) amine of 8.7 is in agreement with the previous results of Anthonsen and Smidsrød[11] for chito-oligomers with varying F_A (0.0 and 0.5) and DP_n (4 and 14), and recently published data.[12]

Characterisation of chitosan branched with acetaldehyde or D-glucose.—The fully de-*N*-acetylated chitosan ($F_A < 0.001$, $DP_n = 25$) was substituted with D-glucose (DS 0.20) and acetaldehyde (DS 0.20). The resonances in the ^{1}H NMR spectra were assigned by COSY (data not shown), and the pK_a values determined from the ^{1}H NMR titration curves, as shown in Fig. 3. From Figs. 3 and 4 it is evident that the secondary amines have different pK_a values depending on the nature of the substituent (see Table 1). The pK_a and pK_{int} values of the ethyl-substituted secondary amines were determined to 6.6 and 8.4, respectively, which are essentially the same as for the primary underivatised amines. However, the pK_a values of the secondary amines substituted with glucose and the trimer **A**–**A**–**M** were determined to 5.5 and 5.0–5.2, respectively, i.e., significantly lower than the primary amines and the ethyl-substituted secondary amines. A possible explanation for the lower pK_a values is that some of the hydroxyl groups of the glucose and trimer branches can form hydrogen bonds to the nitrogen, making it more acidic.[31]

Neutral-solubility of the branched chitosans.—The chitosan branched with the trimer **A**–**A**–**M** (DS 0.40)

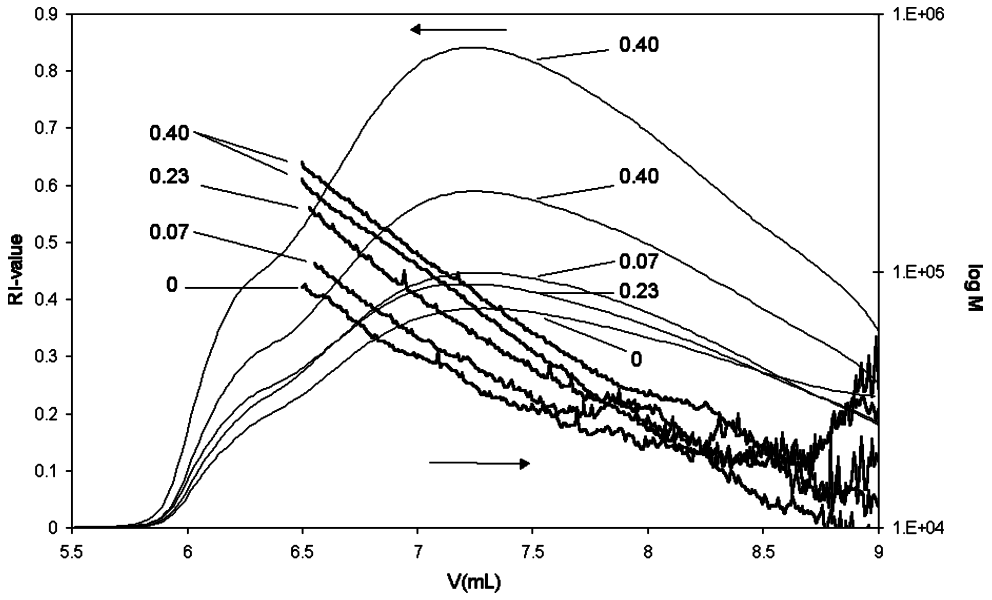


Fig. 5. Molecular weight distribution and concentration profiles (RI) for the chitosans *N*-alkylated with the trimer **A**–**A**–**M** to different degrees of substitution (DS) obtained by SEC-MALLS (0.25–0.50 mg injected, 0.2 M ammonium acetate, pH 4.5, 25 °C).

was soluble at low pH values, and did not precipitate upon increasing the pH value, in contrast to the starting chitosan ($DP_n = 25$, $F_A < 0.001$) which precipitated above pH 6.7 (see Table 1). A general increase in neutral-solubility was also found by Casu et al.,[20] when branching chitosan with similar oligosaccharides, although no study of the effect of the pH was performed. This means that the solubility of chitosans in aqueous solution over the entire pH range can be enhanced by the introduction of oligosaccaride branches.

SEC-MALLS.—The comb-shaped chitosans (**A**–**A**–**M** substituted on the low molecular weight chitosan with $F_A < 0.001$ and $DP_n = 25$) were studied by SEC-MALLS (size-exclusion chromatography with a multi-angle laser light scattering detector). The chromatograms and the corresponding calibration plots of the molecular weight (M) versus elution volume (semilogaritmic scale) are shown in Fig. 5. The elution profiles were nearly identical for the different chitosans, whereas the calibration plots differed, with a shift towards higher M with increasing DS. This can be rationalised in the following way: because of the stiffness of the β-(1→4)-linked chitosan chains, the short oligomers (DP around 25) will behave as rigid rods. When introducing trimer branches, these contribute negligibly to the length of the polymer, and therefore also insignificantly to the molar hydrodynamic volume, which formed the basis for retention in SEC. However, because of the increase in mass, branching of chitosan oligomers will therefore lead to a decrease in the intrinsic viscosity ([η]).

4. Conclusions

-Branched chitosans were prepared by reductively alkylating the trimer (**A**–**A**–**M**) onto the 2-amino group under homogeneous conditions. Degree of substitution of 0.07, 0.23 and 0.40 were obtained.
-The secondary amine at the branching point was found to have a lower pK_a and pK_{int} (5.0–5.2 and 7.3–7.4, respectively) than the unsubstituted primary amine (6.6 and 8.7, respectively).
-The 40% branched chitosan was found to be soluble at all pH values.
-SEC-MALLS showed that the branches contributed negligibly to the molar hydrodynamic volume. However, because of the increase in mass, a decrease in the intrinsic viscosity would be expected.

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